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Authors

Eghbal-Ahmadi, M
Hatalski, CG
Avishai-Eliner, S
et al.

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Corticotropin releasing factor receptor type II (CRF_2) messenger ribonucleic acid levels in the hypothalamic ventromedial nucleus of the infant rat are reduced by maternal deprivation

MARIAM EGHBAL-AHMADI, **CAROLYN G. HATALSKI**, **SARIT AVISHAI-ELINER**, and **TALLIE Z. BARAM**
Departments of Anatomy & Neurobiology (MEA, CGH, TZB) and Pediatrics (SAE, TZB) University of California Irvine, Irvine, CA 92697-4475

Abstract

The stress neurohormone corticotropin releasing factor (CRF) activates at least two receptor types. Expression of corticotropin releasing factor receptor type II (CRF_2) has been demonstrated in the hypothalamic ventromedial nucleus (VMH) of the adult and developing rat, but the physiological functions of VMH-CRF_2 have not been elucidated. The VMH has been documented as an important participant in the regulation of food intake and its interactions with the hypothalamic-pituitary-adrenal axis and circadian rhythms. Regulation of VMH-CRF_2 may thus play a role in the interplay of physiological alterations in metabolic state with the neuroendocrine and anorexic effects of CRF. This study determined the regulation of CRF_2-mRNA expression in infant rats by the physiological consequences of maternal deprivation, i.e., fasting and stress. Using *in situ* hybridization, maternally deprived pups had an average 62% reduction of VMH-CRF_2-mRNA levels compared with stress-free controls. Maternal deprivation also resulted in elevated plasma corticosterone levels (3.8 ± 0.3 vs. 1.3 ± 0.1μg/dl) and an average 5.7% body weight loss. This study demonstrates that maternal deprivation, via fasting and HPA activation, leads to a dramatic decrease of CRF_2-mRNA levels in the VMH. These results are consistent with a role for CRF_2 activation in mediating some of the complex interactions of CRF (or urocortin) with regulation of food intake in the developing rat.

CRF is the key mediator of the neuroendocrine response to stress (1). Two major receptors activated by CRF have been characterized, type I (CRF_1) and type II (CRF_2) (2–5). The role of CRF_1 has been extensively investigated: This receptor is a primary mediator of the actions of CRF in the hormonal response to stress, resulting in ACTH release from the pituitary (1,6) and the activation of this receptor in the amygdala is required for the anxiogenic effects of CRF (7). However, the physiological roles of CRF_2 have not been fully defined (2). Furthermore, the relative roles of the two receptor types in mediating certain actions of CRF, in particular the profound anorexia induced by the central administration of this peptide, have not been clarified. Recent evidence has suggested that CRF may decrease food intake via mechanisms including CRF_2 activation (8). This notion is supported by the actions of urocortin, a CRF related peptide with a higher binding affinity for CRF_2, which has been found to be more potent than CRF itself in depressing food intake (8,9). The location of CRF_2 which putatively mediate the anorexic effects of CRF or related ligands has also not been resolved. Within the hypothalamus, mRNA for CRF_2 is present in both the paraventricular nucleus (PVN) and the VMH, and the latter nucleus contains far higher concentrations of CRF_2-mRNA (10). In addition, the VMH has been shown to be involved in satiety control as well as the integration of signals of the circadian rhythm, stress and food intake (11,12). However, CRF infusions into discrete hypothalamic nuclei have implicated the PVN as the site of the anorexic effects of CRF (13). This study aimed to examine the effects of the physiological stressor of maternal deprivation, which includes

both fasting and hypothalamic-pituitary-adrenal axis (HPA) activation, on CRF_2 mRNA levels in the VMH, as means for studying the potential role of this receptor in modulating the homeostatic interactions of CRF/urocortin, food intake and stress.

Materials and Methods

Animals and tissue preparation

Timed-pregnancy Sprague-Dawley rats (Zivic-Miller, Zelienople, PA) were maintained in NIH-approved animal facilities on a 12-hour light/dark cycle with access to unlimited lab chow and water. Delivery was verified at 12-h intervals and the date of birth was considered day 0. Litters were culled to 12 pups and mixed among experimental groups. On the morning of postnatal day 8, littermates were separated into a maternally deprived group (n=7) and a non-deprived group (n=4) left undisturbed in their home cage for 24h. The maternally deprived pups were kept on a warm pad maintaining a euthermic core temperature (33–34°C), under a 12h light/dark cycle. Pups were weighed as a group before and after deprivation. On the morning of postnatal day 9, the non-deprived pups were weighed, and sacrificed within 3 minutes of their initial disturbance. For all pups trunk blood was collected for measurement of plasma corticosterone (CORT) levels and brains were rapidly dissected and frozen. Twenty micron coronal brain sections were cut, mounted on gelatin-coated slides and stored at −80°C. All experimental procedures were approved by the institutional animal care committee and conformed to NIH guidelines. Plasma CORT levels were analyzed using a commercial radioimmunoassay kit (ICN, Irvine, CA) as previously described (14).

Synthesis and preparation of the riboprobe

A plasmid containing the 461 base-pair fragment of CRF_2 cDNA (kindly provided by Dr. Lovenberg, Neurocrine Biosci., Inc.) was linearized with Hind III (5). Radioactive antisense cRNA was synthesized by incorporating [α-^{35}S]-CTP (New England Nuclear, Boston, MA), using T3 RNA polymerase (30U, Promega, Madison, WI) according to manufacturer's instruction. After incubating at 37°C, 3U of RNase-free DNase (RQ1-DNase, Promega) was added for 15 minutes at 37°C. Integrity of each transcript was determined by acrylamide gel electrophoresis. The probe was subjected to alkaline hydrolysis and purified by column chromatography (Select-D[RF], 5 prime-3 prime, Boulder, CO). The specific activity of each probe was 1–3 $\times 10^6$ cpm/mg. Probe specificity to CRF_2- mRNA has been established (5).

In situ hybridization histochemistry (ISH)

Sections were brought to room temperature, air dried and fixed in fresh 4% buffered paraformaldehyde for 20 minutes, followed by dehydration and rehydration through graded ethanols (15). Subsequently, sections were exposed to 0.5% acetic anhydride in 0.1M triethanolamine (pH = 8) for 5 minutes and were dehydrated through graded ethanols. Sections were air dried and prehybridized for 1 hour at 55°C in a humidity chamber. Sections were hybridized overnight at 55°C with 1×10^6 cpm of ^{35}S-labeled ribonucleotide probe in hybridization buffer containing 50% formamide, 5X SET, 0.2% SDS, 5X Denhardts solution, 0.5 mg/ml sheared salmon sperm DNA, 25ug/ul yeast tRNA, 100mM Dithiothrietol, and 10% Dextran sulfate. Posthybridization, sections were washed in 2X SSC for 5 minutes at room temperature (1X SSC denotes 0.15 M NaCl, 15 mM trisodium citrate buffer, pH=7) and were digested with RNase (200 ug/ml RNase A; Calbiochem, LaJolla, CA) for 30 minutes at 37°C. Sections underwent successive washes (at 55°C) in 2X SSC and IX SSC for 5 minutes, 0.25X SSC for 30 minutes, and in 0.1X and 0.03X SSC for an hour each. Sections were dehydrated through 100% ethanol and were apposed to film (Hyperfilm *B*-Max, Amersham, IL) for 6 to 10 days. Representative sections were then

dipped in NTB2 nuclear emulsion (Eastman Kodak, Rochester, NY) and exposed for 2–3 weeks. Slides were developed (D-19 developer, KODAK) for 2.5 minutes at 16°C, and counterstained using cresyl violet.

Acquisition and quantitative analysis of CRF_2-mRNA ISH signal

Semiquantitative analysis of CRF_2-mRNA was accomplished using the ImageTool analysis software (University of Texas Health Science Center, San Antonio). Films were digitized using a StudioStar scanner (AGFA, resolution 1200×1200 dots per inch). Densities were calibrated using ^{14}C standards and are expressed in μCi/g. The significance of observed quantitative differences among different experimental groups were evaluated using Student's t-test with Welch's correction for unequal variance.

Results and Discussion

Maternal deprivation for 24h resulted in a significant elevation of basal morning plasma CORT levels ($P<0.0001$) (Fig. 1). This finding is consistent with the well documented stressful effects of maternal deprivation, as defined by activation of the neuroendocrine HPA (14,16–18). Looking at the mechanisms by which maternal deprivation provides a stressor, Stanton et al have shown that the HPA activation observed following maternal deprivation is related to both absence of maternal touch and food deprivation (18). Since food deprivation by itself has also been shown to increase plasma CORT (19), the observed maternal-deprivation induced plasma CORT elevation is likely a result of at least two discrete mechanisms. In the current study, 24h of maternal deprivation, associated with fasting, resulted in an average body weight loss of 5.7% in the deprived pups versus a 19% increase in non-deprived littermates. Therefore, in addition to the feeding-independent HPA activation, maternal deprivation also provided a stimulus for the physiological responses to fasting, including release of corticotropin secretagogues (20). Stress- and fasting-induced HPA activation are considered to be mediated by separate mechanisms, involving input from the VMH in the case of food deprivation (12).

CRF_2-mRNA expression in the VMH was markedly decreased in the maternally deprived pups (Fig. 2). Quantitation of the relative abundance of VMH-CRF_2-mRNA expression revealed a significant decrease (62%; $p< 0.005$) in the deprived rats as compared to controls. The results of this study thus provide evidence that physiological components of maternal deprivation, such as stress and starvation, down-regulate the levels of CRF_2-mRNA in the VMH of infant rats. A number of mechanisms may be involved in this reduction of receptor levels after a complex manipulation such as maternal deprivation.

Decreased CRF_2-mRNA in the VMH of maternally deprived rats may be due to ligand-induced receptor down-regulation as would be expected in the presence of enhanced CRF secretion (21). Previous studies have documented reduced CRF levels in the median eminence (ME) following a 24h period of maternal deprivation, suggesting that CRF is indeed released into the circulation in response to the stress of maternal deprivation (22). The possibility that CRF originating in the ME activates CRF_2 in the VMH requires an anatomical connection between these two regions. A vascular, venous connection between the ME and the VMH has been documented, which may transport CRF (23, 24). Potentially, a stress-induced influx of CRF to the VMH from the ME may activate CRF_2 and result in a compensatory down-regulation of CRF_2-mRNA expression. Teleologically, limiting the anorexic actions of CRF during a state of food-deprivation would be beneficial to the starving organism. A role for CRF in anorexia is suggested from studies demonstrating that central administration of CRF decreases food intake (2) and prevents weight gain in genetically obese rats (25).

In addition to stress, the fasting associated with maternal deprivation may influence CRF secretion from the PVN and alter CRF levels at the site of the CRF_2 receptors in the VMH. The VMH has been shown to alter the secretory function of the PVN, Both direct and indirect neuronal connections (26) may permit the transmission of signals from the VMH to the CRF-secreting neurons in the PVN (27). Thus, fasting would trigger signaling from the VMH to the PVN, inducing CRF secretion. Since CRF synthesis, measured by CRF-mRNA levels in the PVN are reduced or unchanged after food deprivation, enhanced CRF secretion would be expected to occur only within the first hours or days, as in this maternal deprivation paradigm, and not in conditions of chronic weight loss (20). As discussed above, increased activation of VMH-CRF_2 by CRF may lead to down regulation of the expression of this receptor.

Alternatively, the fasting and loss of body weight associated with maternal deprivation may more directly regulate CRF_2 expression in the VMH, independent from activation of the receptor by its physiological ligand. A significant body of literature supports the role of the VMH in regulating food ingestion (28). For example, VMH lesions increased food intake acutely and lead to obesity (12). Food intake and the metabolic signals associated with eating may directly or indirectly regulate CRF_2 expression in the VMH. CRF_2 levels may be involved in the signaling mechanisms from the VMH to the PVN, to influence HPA activation (12, 27). Furthermore, reduced receptor expression in a state of food-seeking may function to limit the ability of CRF (or related ligands) to activate CRF_2 and induce anorexia. In support of a down-regulation of CRF_2-mRNA expression in states of increased food-seeking, Richard et al. have demonstrated, using a genetic model of obesity, that CRF_2-mRNA levels in the VMH were ~ 40% lower in obese (fa/fa) vs. lean (fa/?) individuals fed *ad libitum* (29). Although food-intake was not documented in that study, the marked obesity of the (fa/fa) rats (average weight of 237 gm vs. 175 gm in the controls) suggests increased consumption of food by these rats.

The discussion above assumes that the anorexic effects of CRF or the closely related ligand, urocortin, are mediated by the activation of CRF_2. Strong support for this notion is provided by the marked anorexic effects of urocortin, a CRF-related peptide with much greater affinity for CRF_2 than that of CRF itself. (8, 11, 30). Whether activation of CRF_2 in the PVN or in the VMH is required for anorexia has not been fully resolved. Immuno-targeting and ablation of CRF-containing cells in the PVN abolishes the anorexic effects of endogenous CRF, but the PVN may simply be the origin of the CRF which activates CRF_2 in the VMH (see above). Local injection studies have demonstrated that CRF infusion into the PVN results in significantly decreased food intake, but an equal amount of CRF injected into the VMH does not significantly decrease food intake (13). However, the major receptor type in the VMH, CRF_2 has a much lower affinity for CRF than the major receptor type in the PVN, CRF_1. Thus higher doses of CRF may be needed to cause anorexia via activation of CRF_2 in the VMH.

Of note, a recent report of the up-regulation of urocortin, a putative ligand of CRF_2, by salt-loading in adult rats (31) may be pertinent to the down-regulation of CRF_2 found in this study. Maternal deprivation may lead to some dehydration and increased osmolarity in separated pups. If the hyperosmolarity-induced elevation of urocortin occurs also in suckling rats, then the increased levels of this ligand may contribute to the down-regulation of CRF_2 mRNA.

In summary, 24h of maternal deprivation result in dramatic reduction of CRF_2-mRNA levels in the VMH of the developing rat. The maternal deprivation paradigm provides a complex physiological and psychological stressor, as well as starvation. Synergistic interactions between elements of stress and of food-intake regulation may account for the marked

reduction of CRF_2-mRNA in the VMH, and this reduction is consistent with the involvement of CRF_2 in the interaction of food-intake regulation with the stress response of the developing rat.

Acknowledgments

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References

1. Vale W, Spiess J, Rivier C, Rivier J. Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin. Science. 1981; 213:1394–1397. [PubMed: 6267699]
2. Behan DP, Grigoriadis DE, Lovenberg T, Chalmers D, Heinrichs S, Liaw C, De Souza EB. Neurobiology of CRF receptors and CRF-binding protein: implications for the treatment of CNS disorders. Mol Psychiatry. 1996; 1:265–277. [PubMed: 9118350]
3. Chang CP, Pearse RV, O'Connell S, Rosenfeld MG. Identification of a seven transmembrane helix receptor for CRF and sauvagine in mammalian brain. Neuron. 1993; 11:1187–1195. [PubMed: 8274282]
4. Perrin MH, Donaldson CJ, Chen R, Lewis KA, Vale WW. Cloning and functional expression of a rat brain CRF receptor. Endocrinology. 1993; 133:3058–3061. [PubMed: 8243338]
5. Lovenberg TW, Liaw CW, Grigoriadis DE, Clevenger W, Chalmers DT, De Souza EB, Oltersdorf T. Cloning and characterization of a functionally distinct CRF receptor subtype from rat brain. Proc Natl Acad Sci U S A. 1995; 92:836–840. [PubMed: 7846062]
6. Sawchenko PE, Imaki T, Potter E, Kovacs K, Imaki J, Vale W. The functional neuroanatomy of CRF. Ciba Found Symp. 1993; 172:5–21. [PubMed: 8491094]
7. Rassnick S, Heinrichs SC, Britton KT, Koob GF. Microinjection of a CRF antagonist into the central nucleus of the amygdala reverses anxiogenic-like effects of ethanol withdrawal. Brain Res. 1993; 605:25–32. [PubMed: 8467387]
8. Spina M, Merlo-Pich E, Chan RK, Basso AM, Rivier J, Vale W, Koob GF. Appetite-suppressing effects of urocortin, a CRF-related neuropeptide. Science. 1996; 273:1561–1564. [PubMed: 8703220]
9. Vaughan J, Donaldson C, Bittencourt J, Perrin MH, Lewis K, Sutton S, Chan R, Turnbull AV, Lovejoy D, Rivier C, Rivier J, Sawchenko PE, Vale W. Urocortin, a mammalian neuropeptide related to fish urotensin I and to CRF. Nature. 1995; 378:287–292. [PubMed: 7477349]
10. Chalmers DT, Lovenberg TW, De Souza EB. Localization of novel corticotropin-releasing factor receptor (CRF2) mRNA expression to specific subcortical nuclei in rat brain: comparison with CRF1 receptor mRNA expression. J Neurosci. 1995; 15:6340–6350. [PubMed: 7472399]
11. Richard D. Involvement of CRF in the control of food intake and energy expenditure. Ann N Y Acad Sci. 1993; 697:155–172. [PubMed: 8257008]
12. Dallman MF. Viewing the ventromedial hypothalamus from the adrenal gland. Am J Physiol. 1984; 246:R1–R12. [PubMed: 6320667]
13. Krahn DD, Gosnell BA, Levine AS, Morley JE. Behavioral effects of corticotropin-releasing factor: localization and characterization of central effects. Brain Res. 1988; 443:63–69. [PubMed: 2834018]
14. Avishai-Eliner S, Yi SJ, Newth CJ, Baram TZ. Effects of maternal and sibling deprivation on basal and stress induced HPA components in the infant rat. Neurosci Lett. 1995; 192:49–52. [PubMed: 7675308]
15. Baram TZ, Lerner SP. Ontogeny of corticotropin releasing hormone gene expression in rat hypothalamus-comparison with somatostatin. Int J Dev Neurosci. 1991; 9:473–478. [PubMed: 1685845]
16. Levine S, Huchton DM, Wiener SG, Rosenfeld P. Time course of the effect of maternal deprivation on the HPA in the infant rat. Dev Psychobiol. 1991; 24:547–558. [PubMed: 1773913]

17. Rosenfeld P, Wetmore JB, Levine S, Menzaghi F, Rassnick S, Heinrichs S, Baldwin H, Pich EM, Weiss F, Koob GF. Effects of repeated maternal separations on the adrenocortical response to stress of preweanling rats The role of CRF in the anxiogenic effects of ethanol withdrawal. Ann N Y Acad Sci. 1994; 739:176–184. [PubMed: 7832471]
18. Stanton ME, Gwosdow AR, Levine S. Maternal deprivation potentiates pituitary-adrenal stress responses in infant rats. Behavioral Neuroscience. 1988; 102:692–700. [PubMed: 3196438]
19. Dallman MF, Strack AM, Akana SF, Bradbury MJ, Hanson ES, Scribner KA, Smith M. Feast and famine: critical role of glucocorticoids with insulin in daily energy flow. Front Neuroendocrinol. 1993; 14:303–347. [PubMed: 8258378]
20. Schwartz MW, Seeley RJ. Neuroendocrine responses to starvation and weight loss. New Eng J Med. 1997; 336:1802–1811. [PubMed: 9187072]
21. Rabadan-Diehl C, Kiss A, Camacho C, Aguilera G. Regulation of messenger ribonucleic acid for corticotropin releasing hormone receptor in the pituitary during stress. Endocrinology. 1996; 137:3808–3814. [PubMed: 8756551]
22. Pihoker C, Owens MJ, Kuhn CM, Schanberg SM, Nemeroff CB. Maternal separation in neonatal rats elicits activation of the HPA: a putative role for CRF. Psychoneuroendocrinology. 1993; 18:485–493. [PubMed: 8265736]
23. Ambach G, Palkovits M. Blood supply of the rat hypothalamus. V The medial hypothalamus (nucleus ventromedialis, nucleus dorsomedialis, nucleus perifornicalis). Acta Morphol Acad Sci Hung. 1977; 25:259–278. [PubMed: 617771]
24. Ambach G, Palkovits M, Szentagothai J. Blood supply of the rat hypothalamus. IV Retrochiasmatic area, median eminence, arcuate nucleus. Acta Morphol Acad Sci Hung. 1976; 24:93–119. [PubMed: 1031571]
25. Rohner-Jeanrenaud F, Walker CD, Greco-Perotto R, Jeanrenaud B. Central CRF administration prevents the excessive body weight gain of genetically obese (fa/fa) rats. Endocrinology. 1989; 124:733–739. [PubMed: 2536319]
26. Canteras NS, Simerly RB, Swanson LW. Organization of projections from the ventromedial nucleus of the hypothalamus: a phaseolus vulgaris-leucoagglutinin study in the rat. J Comp Neurol. 1994; 348:41–79. [PubMed: 7814684]
27. Choi S, Horsley C, Aguila S, Dallman MF. The hypothalamic ventromedial nuclei couple activity in the HPA axis to the morning fed or fasted state. J Neurosci. 1996; 16:8170–8180. [PubMed: 8987842]
28. Dallman MF, Akana SF, Strack AM, Hanson ES, Sebastian RJ. The neural network that regulates energy balance is responsive to glucocorticoids and insulin and also regulates HPA axis responsivity at a site proximal to CRF neurons. Ann N Y Acad Sci. 1995; 771:730–742. [PubMed: 8597446]
29. Richard D, Rivest R, Naimi N, Timofeeva E, Rivest S. Expression of CRF and its receptors in the brain of lean and obese Zucker rats. Endocrinology. 1996; 137:4786–4795. [PubMed: 8895348]
30. Heinrichs SC, Menzaghi F, Merlo-Pich E, Hauger RL, Koob GF. CRF in the paraventricular nucleus modulates feeding induced by neuropeptide Y. Brain Res. 1993; 611:18–24. [PubMed: 8518948]
31. Hara Y, Ueta Y, Isse T, Kabashima N, Shibuya I, Hattori Y, Yamashita H. Increase of urocortin-like immunoreactivity in the rat hypothalamo-neurohypophysial system after salt loading and hypophysectomy. Neurosci Lett. 1997; 227:127–130. [PubMed: 9180220]

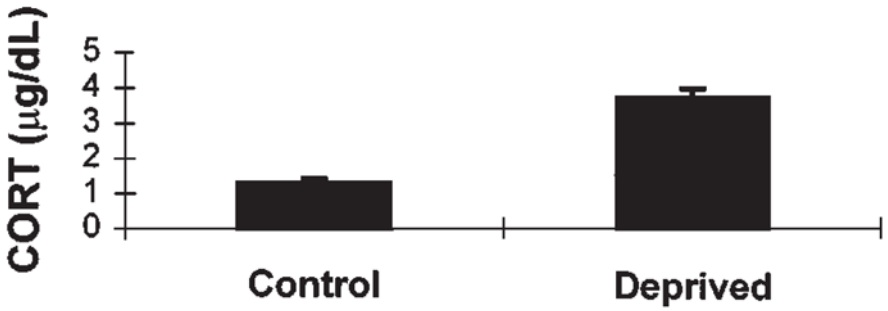

Fig. 1.

Elevation of basal plasma corticosterone (CORT) levels in maternally deprived 9 day old rats. 24h of maternal deprivation resulted in a significant increase ($p<0.0001$) in morning plasma CORT levels compared with non maternally deprived controls.

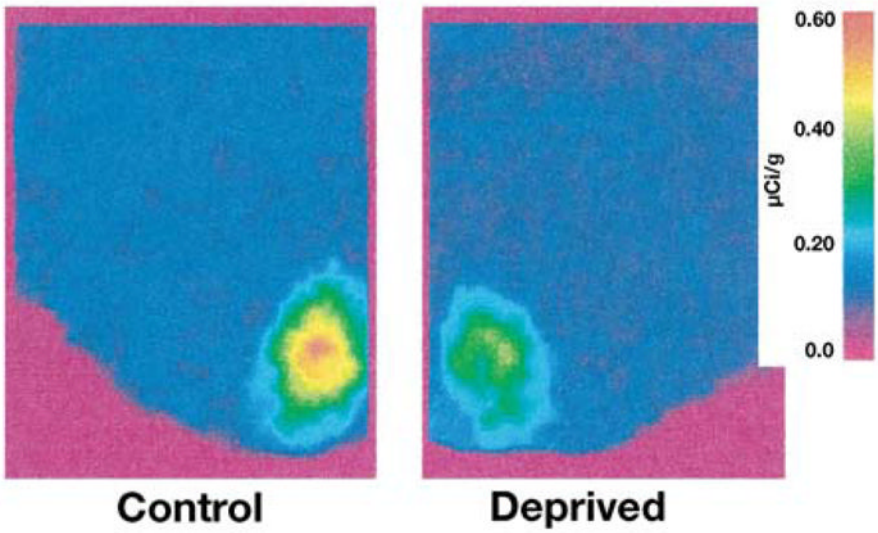

Fig. 2.

Computer analyzed false color images showing CRF_2 - mRNA levels in the VMH of stress-free (control) and maternally deprived rats. Red and yellow colors represent maximum ^{35}S riboprobe density as shown in the scale.

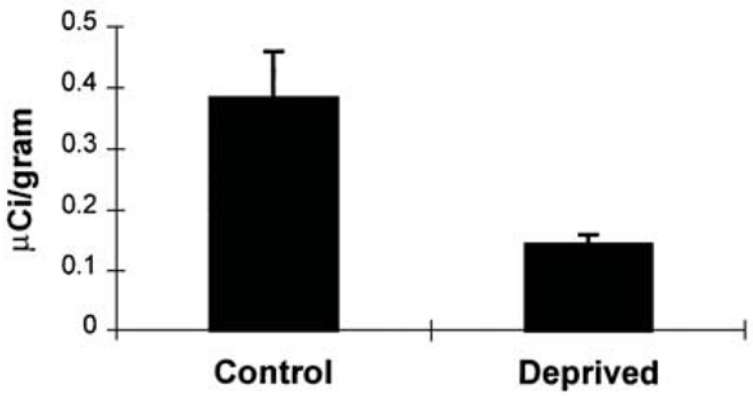

Fig. 3.

Semiquantitative analysis of CRF_2-mRNA expression within the VMH of maternally deprived rats. Bars represent the mean CRF_2-mRNA expression in the VMH of 9 day old rats maternally deprived for 24h or non-maternally deprived controls with error bars illustrating the standard error of the mean. A significant decrease in CRF_2-mRNA abundance was observed as a consequence of maternal deprivation ($p<0.005$).